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Analysis of the Pathomechanism of Migraines with a Focus on Current Treatment Plans and the Role of the Neuropeptide CGRP

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ANALYSIS OF THE PATHOMECHANISM OF MIGRAINES WITH A FOCUS ON CURRENT TREATMENT PLANS AND THE ROLE OF THE NEUROPEPTIDE CGRP

by

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ABSTRACT

Migraines are a type of headache that specifically act on only one side of the head, although about 30% of patients with migraine may experience a bilateral headache. Migraine is a brain disorders that typically involve issues of the typical sensory processing that takes place in the brainstem. Possible causation has been linked to issues in blood vessels, blood flow, and oxygen levels in the brain. Migraine can be described in three phases, and common throughout the three phases is the importance of the neuropeptide CGRP and its role in migraine pathogenesis. CGRP increases in plasma have been linked to migraine headaches, and specific treatment plans have been tailored to account for this. CGRP is a vasodilator that causes dilation of cranial blood vessels and can lead to possible neurogenic inflammation in the periphery of its release while activating the pain pathway in the brainstem. The primary treatment for migraines is currently drugs from the triptan family and NSAIDs, as well as prophylactic drugs including antiepileptic drugs, beta-blockers, and Ca^{2+} channel blockers. The experiment conducted for this project aimed to determine the effects of a specific CGRP polyclonal antibody and CGRP receptor antagonist when it is with capsaicin, which stimulates sensory nerves. In an ex-vivo experiment using cell culture medium, the dura mater of mice is given either rabbit polyclonal antibody or a CGRP receptor antagonist or both, and then is challenged with capsaicin. CGRP positive (expressing) fibers and nerve terminals are examined under a fluorescent microscope in the dura mater of the mice.

DEDICATIONS

For Dr. Samsam, thank you for believing in me and being not only a great faculty mentor, but also an ideal role model.

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INTRODUCTION

Migraine is a specific type of headache that involves a unilateral pulsing pain. This pain typically affects only one side of the head, although about 30% of patients may experience a bilateral headache. Migraine can be described as a brain disorder in the brainstem that involves issues of typical sensory processing present in the brainstem. The typical duration of a migraine is from 4 to 72 hours. This is typically accompanied with nausea, disturbed vision, vomiting, photophobia (hypersensitivity to light), and phonophobia (hypersensitivity to sound)[1]. Migraines are split into two different classes: the common and classic migraine. The majority of migraine is classified as common migraine. Migraine can be classified as with and without aura; common migraines are not accompanied with aura. Classic migraine are the second class that is accompanied by neurological symptoms known as aura, which are disturbances that are visual, motor, and sensory[2]. The classic migraine comprises around 15% of total migraines and is thus the minority class of migraines[1]. For both types of migraines, a 3/1 female to male ratio is observed. The method of action of aura is through potential cortical spreading depression (CSD). CSD is a wave of neuronal activity that starts from the occipital lobe to the frontal lobe. CSD leads to a neuronal inhibition and is accompanied by lower cerebral blood flow[2].

The etiology of migraine involves many different issues including the abnormality in the cerebral blood vessel walls, impairment in blood flow (that could be due to issues in vessel contraction), abnormal platelet numbers, or varying brain oxygenation or even

metabolism levels[2]. Migraine can be caused in response to a single or combined effect of all of these issues, and varies on the specific case of migraine displayed and on the individual displaying it. However, constant throughout all of these varying etiological factors is the involvement of the blood vessels, either intracranial or extracranial, as well as information communicated by the sensory nerve fibers to the central nervous system[2]. Moreover, studies conducted through positron emission tomography (PET) have indicated that the activation of the brainstem and brain is involved in initiating migraines and is constant both with and without aura[3]. Alcohol, particular food or odors, and weather may also be involved in the initiation of migraines and may serve as potential trigger factors, depending on the particular individual and situation[2].

This thesis is a brief review of the pathophysiology and treatment of migraine headaches by focusing on the most involved neuropeptide, the calcitonin gene related peptide (CGRP), in addition to new drugs still in clinical trials against CGRP release from sensory neurons of antagonizing its receptors in the brainstem and vessels. I will present the concept of the anti-CGRP treatment of migraine along with preliminary experiments we conducted on ex-vivo mouse dura mater in cell culture medium at 37 degrees Celsius in an incubator. Sensory nerve fibers and meningeal vessels were analyzed upon stimulation with capsaicin following treatment with CGRP antibody and/or CGRP antagonists.

PATHOPHYSIOLOGY OF MIGRAINE

The exact pathomechanism of migraine is not known. Migraine is a brain disorder, system failure and sensory dysmodulation of the typical sensory processing that takes place of the brainstem[4]. In a typical migraine, the excitatory events occur prior to the neurovascular events, and after the occurrence of the neurovascular events, pain is evident in the patient. Positron emission tomography (PET) studies have determined that the initial phase of migraine begins due to neuronal hyperexcitability as well as activation of the brainstem, brain[5], dorsal rostral pons[6], dorsal pons, dorsal midline pons[7], and hypothalamus, among others[8].

There is an endogenous pain-controlling center called the periaqueductal gray (PAG) matter in the brainstem. The PAG is location of the origin of noradrenergic and serotonergic descending antinociceptive fibers. These fibers end at different levels of the spinal cord and brainstem, and at the various levels at which the fibers end, opioid-containing interneurons are activated. In several patients during migraines, this pain inhibiting system has had several reported dysfunctions on the nociceptive response to trigeminal stimulation[9]. Related theories describe migraine attacks as a result of a top down dysfunctional process. This begins in a hyperexcitable and hypoenergetic brain, and continues downstream from the frontal lobe to incorrectly activated nuclei that are in the pain matrix, describing the “top-down” of the process[10, 10a].

Many different theories exist regarding the etiology of migraine, most revolving around potential dysfunction involved in the cerebral blood vessel walls, impairment of blood flow (which may be related to abnormal vessel contractility), circulating vasoactive

material, platelet issues, and even issues related to brain metabolism and oxygenation[11]. Migraines may involve extracranial and/or intracranial blood vessels, and nociceptive information from these blood vessels use sensory nerve fibers to transport sensory information[2]. Vascular involvement may not be, however, a particular trigger factor for migraines, but is instead linked with the pain that is felt by the patient during a typical migraine. Other factors proposed were changes in serotonin levels (5-HT), issues with mitochondrial metabolism, decreased blood tissue magnesium, changes in ion transport across the membrane of the cell, and genetics[12]. There are also several other factors that can initiate migraine in some patients, which can vary from individual to individual and on the particular situation. These particular trigger factors include weather changes, odors and foods, alcohol, and decreased levels of estrogen[13].

Though vascular involvement isn't described as a trigger factor for migraines, it is instead involved with the pain related through migraine, related, in particular, to the dilation of cranial blood vessels, which includes arteriovenous anastomotic shunts[26]. Dilation in these vessels, including these anastomotic shunts, takes place as a response to activation of the trigemino-vascular system, which is theorized to cause the pain associated with headaches[14]. In addition, patients with cranial arteriovenous malformations have reported a greater incidence of migraines, and it has been determined that correcting these related issues has led to reported pain decrease[26].

A specific triggering factor that is observed to be important in migraine is channelopathies, which is a mutation in specific channels that leads to variation in transport along the membrane transport of different ions. Genetic defects that involve

channelopathies in the P/Q type calcium channel gene, the Na+/ K+ pump, and/or the Nav1.1 sodium channel have been linked to migraine and are examples of specific channelopathies[16, 16a]. All of these genetic defects have been associated with the pathophysiology of the familial hemiplegic migraine (FHM), which are classified as classic migraines and are accompanied by aura. Mutations in these transport related substances have made patients more susceptible to migraines.

Three Phases of Migraine Headaches

A typical migraine can be described as proceeding through three phases: 1) the trigger phase, which starts through neuronal hyperexcitability as well as activation of the brain, 2) the aura phase, which may include cortical spreading depression (CSD), and 3) the headache phase, which involves activation and sensation of the trigeminal nerve, and to a lesser extent other nerves, as well as cranial vasodilatation. The symptoms displayed in the aura and the headache phases are described through the neurovascular theory and are important in the development of migraine treatment. There are, however, two general phases that take place following the end of the migraine, the resolution and the postdrom phases[18]. These phases aren't part of the three main phases of migraine.

During the trigger phase, the first main phase of migraine, the activation of the brainstem takes place, which continues and is unchanged even when sumatriptan (an antimigraine drug) is used. The possible genetic issues discussed previously regarding membrane transport are manifested in this phase and may alter the response threshold

to triggers specific to migraines. These genetic issues are related to channelopathies that were previously discussed.

The aura phase takes place after the trigger phase, during which patients may display aura, which is described as the symptoms that are experienced while suffering from a classic migraine. In general, the symptoms displayed in the headache and aura phases can be described through the neurovascular theory. Aura is present in 30% of migraine patients and involves symptoms such as visual disturbances, which typically occur about an hour before the onset of the headache, flashing lights, scotomas, and zigzags (fortifications), as well as photophobia, andphonophobia[20].

Aura could possibly be due to cortical spreading depression (CSD), a spike activity that takes place in the occipital cortex and goes anteriorly, eventually leading to neuronal inhibition[21]. This wave is then followed by a secondary moderate regional oligemia, which is described as a decrease in cerebral blood flow[21]. Thus, it is believed that the aura phase takes place through a continuous wave present in the astroglial gap-junction coupled network. However, it is also possible that the aura phase comes about because of neuronal dysfunction, which is caused by CSD and not due to ischemia[22].

Channlopathies have also been linked to various types of familial hemiplegic migraines (FHM) that are present with aura. These channelopathies are believed to make the patient more likely to suffer from migraine[23]. This increase can be attributed to: the increase in synaptic glutamate release (FHM1), decreased removal of K+ and glutamate from the synaptic cleft (FHM2), or excessive extracellular K+ (FHM3)[23]. An

inhibitor of the gap junction/ CSD in the Phase II Clinical Trial, Tonabersat, prevents issues associated with migraine aura but has no consequence on attacks that aren't associated with aura. Many anti-migraine drugs (prophylactic) aim to suppress CSD as their method of action. Examples of such drugs are methysergide, amitriptyline, propranolol, valproate, and topiramate[24].

The exact transition of the aura phase to the headache phase is unknown. Headache phases usually take place in the frontotemporal region of the brain. The pain in migraine headaches is unilateral and described as throbbing, which is the typical pain that has been associated with migraine. Increased intracranial pressure leads to peripheral sensitization of the meningeal sensory fibers, and it is believed to lead to the throbbing description of the pain. The pain has reportedly increased by physical activities, bending, and coughing, and others that may vary from individual to individual[25].

The extracranial and intracranial blood vessels, as well as the meninges and their innervations, in addition to the cerebral sinuses, are the main structures responsible for transmitting harmful stimuli[2]. The trigeminovascular system, which is composed of the trigeminal nerves and the cerebral blood vessels as its target innervation, conveys the pain to the brainstem activating pathways[11].The headache phase of migraine is believed to develop as a consequence of a primary disturbance in the trigeminovascular neurons as well as their central or peripheral processes based on the trigeminovascular theory[2].

Several neuropeptide contents of the trigeminovascular system have been implicated in migraine, which lower threshold and depolarize sensory nerve fibers by binding to the specific receptors on the nerve fibers and vessels associated with the system[26]. Among these, in an experiment conducted by Fribergetal and Gallavetal, the calcitonin gene related peptide (CGRP) was the only neuropeptide that was increased clinically in the blood of some migraine patients[26]. CGRP and other inflammatory mediators (SP and NKA) cause dilation of the blood vessels as well as inflammation. However, experiments have indicated that migraines begin without dilation of the middle cerebral artery (MCA), and exogenous CGRP has been found to instead dilate the human middle meningeal artery (MMA)[27]. In line with this finding is the activity of sumatriptan, which has been found to lead to its antinociceptive action by constricting the MMA but not the MCA[28].

CALCITONIN GENE RELATED PEPTIDE

CGRP is an important 37 amino acid neuropeptide that was discovered by RNA transcripts[26]. These transcripts are from the calcitonin gene and alternative processing leads to different mRNAs that encode the hormone calcitonin[27]. CGRP plays an important role in primary sensory neurons in which it is localized, specifically through activation of the trigeminal system[29]. Its associated areas of action have a wide distribution from the peripheral skin, cornea, respiratory and urogenital systems to the terminals close to smooth muscle. CGRP containing neurons and fibers are also found in autonomic ganglia and in parts of the central nervous system and in the cerebral dura[29].

Activation of the trigeminal system as well as tissue stimulation and/or injury causes release of CGRP from the brainstem trigeminal nucleus and spinal cord. Cranial vasodilation, coupled with the activation and sensitization of sensory nerves, has been suggested by several studies to lead to the headache phase[29]. CGRP is a vasodilator of the cranial vessels, and this causes the nerve endings to be disturbed and the nerves to be pinched. This causes more vasoactive material to be released, such as CGRP, which is a vicious cycle resulting in neurogenic inflammation. The method of action has been found to be related to CGRP blocking the release of aldosterone secretion as well as promoting the release of catecholamine, which is done through CGRP Type 1 receptors. This action leads to the vasodilation effect that is associated with CGRP[27].

CGRP has also been found to enhance the activity of another neuropeptide, Substance P (SP), when they are both co-administered in the CNS. This has been

suggested due to CGRP's ability to inhibit an enzyme directly involved in SP degredation[29]. SP has been linked to excitation of the neuronal network, which is where CGRP is believed to control SP degradation. Thus, CGRP release has been shown to have correlations with the duration and intensity of painful stimuli.

The CGRP receptors have been found to contain proteins (RAMPs, or receptor activity modifying proteins) that must be present to move the receptor to the membrane of the cell. There are various forms of RAMP, where RAMP1 moves the functional glycoprotein receptor to the membrane surface to act as a receptor for CGRP[30]. This variant in humans is the rate-limiting factor for the release of CGRP in neurons[31].

Previously, CGRP receptors were separated into 2 CGRP receptor types, however, as of now, experiments have confirmed that there's only 1 CGRP receptor, which is the CGRP1 receptor. There are many components of a CGRP receptor, which include: a transmembrane domain for a receptor activity modifying protein type 1 (RAMP1), a G protein coupled receptor, and a receptor component protein (RCP)[32]. All of these components are necessary to form a working CGRP receptor. For example, RAMP1 is necessary to move G protein coupled receptors that are mature to the cell membrane[70]. It has been determined that the first 7 amino acids on the N-terminal end of the CGRP receptor are necessary to lead to the activation of the receptor, and as such various CGRP receptor antagonists are involved with this end of the protein[32].

CGRP receptors can be found in many different locations in many different types of cells, and aren't just limited to the cardiovascular and nervous system. CGRP receptors are on glia and neurons in the central nervous system, as well as on many

second order neurons, in addition to mast cells that are present inside the dura mater[32]. Various antimigraine treatments act on CGRP release through involvement of CGRP receptors. NSAIDs block the release of CGRP that is promoted through the activation of the prostaglandin receptor. Neuronal 5HT receptors are activated by triptans, which leads to the blockage of CGRP release[33].

Dural surface electrical stimulation has led to a release of CGRP from trigeminal afferents. This leads to vasodilation and an increase of the meningeal blood flow. Nitric oxide (NO) has a synergistic effect along with CGRP on the blood flow, and this theory has been proposed because to the NO-mediated facilitation of CGRP synthesis and release in the trigeminal ganglia neurons[34]. CGRP promoter activity has been increased by overexpression of nitric oxide synthase and NO donors.

Nitric oxide is a vasodilator similar to CGRP that affects the arterial diameter and increases it, which leads too an increase in blood flow throughout the brain, causing a similar role to CGRP in the pathomechanism of migraine[35]. In addition, NO increases the production and exocytosis of CGRP, even when the stimulation is electrical[34]. Accordingly, inhibitors of nitric oxide synthesis prevent CGRP release[37].

Sex hormones in females are also important in the synthesis and the eventual expression of the receptor for CGRP[26]. 17B-estradiol leads to increased neurogenic vasodilation, and the mechanism through which this takes place is suggested through an increase of CGRP. This is believed to be a method by which 17B-estradiol exacerbates migraines in females[38].

CGRP is has also been found with the brain-derived neurotrophic factor (BDNF) in trigeminal neurons, making BDNF a mediator of trigeminal nociceptive plasticity[26]. BDNF is a neurotrophin that regulates factors such as maintenance, differentiation, and survival of various central and peripheral neurons. When BDNF is in low concentrations, this can lead to excitation of neurons in the hippocampus, cerebellum, and cortex[39]. Future studies on the release of BDNF are also potential treatment areas of interest for migraines.

CGRP has also been found to increase plasma protein leakage by various neuropeptides, including SP. The effect of these neuropeptides has been increased by injection of CGRP[40]. It has accordingly been theorized that the co-release of CGRP with these neuropeptides may have additive effects in pain. In addition, CGRP and SP levels have been found to be increase in salivary secretions of patients suffering from migraines[26]. Thus, inhibiting the release of these neuropeptides, specifically CGRP, is an important treatment plan for migraine. As mentioned before, clinically, only CGRP has been detected in the blood of migraine sufferers[26].

CURRENT TREATMENT OF MIGRAINE

Prophylactic treatments are used to treat acute migraine attack, and are currently used as strategies to treat migraine headaches. Treatment methods include the first-line drugs such as triptans and NSAIDS. But also, anti-epileptic drugs, antidepressants, beta-blockers, and natural supplements are used to treat migraines (Table 1)[4]. Acupuncture, as well as other non-drug treatments is used as well.

Medications

Drugs are utilized in the asymptomatic phase between acute attacks where no symptoms are observable while in the prophylaxis of migraine[2]. These drugs include Timolol and Propranolol, which are both beta-blockers. Propranolol has been found to act by inhibiting cortical spreading depression in the aura phase of migraines, and a possible method of action to achieve this has been through the blockage of glutamate release[41]. For patients with depression or sleep issues, Amitriptyline is used, which is a tricyclic antidepressant[40]. Drugs such as Divalproex, an anticonvulsant, and Verapamil, a calcium channel blocker, are also used[2].

Antimigraine Drug	Class	Action
Propranolol	Beta-Blocker (blocks beta-adrenergic receptors)	Acts in asymptomatic phase between attacks
Timolol		
Amitriptyline	Tricyclic Antidepressant	Acts in asymptomatic phase between attacks, used by patients with depression or sleep issues
Divalproex	Anticonvulsant	Acts in asymptomatic phase between attacks
Verapamil	Calcium Channel Blocker	Acts in asymptomatic phase between attacks
Zolmitriptan	Triptan Family [serotonin receptor (5-HT1B/D) agonists]	Act in prodromal phase. Quickly decrease migraine headache onset. Can also constrict coronary arteries
Naratriptan		
Rizatriptan		
Eletriptan		
Almotriptan		
Sumitriptan		
Dihydroergotamine	Derivative of Ergotamine [acting on serotonin receptor (5-HT1B/D) and other receptors]	Vasoconstrictor
Naproxen	Non-steroid anti-inflammatory drugs (inhibit COX-1 and COX-2)	Used in headache phase and help to deal with symptoms
Meclogenamate		
Aspirin		
Domperidone	Anti-emetic drugs	Used to treat nausea in headache phase
Metoclopramide		
Olcegepant	CGRP receptor antagonist	Block central and peripheral CGRP receptors
Telecagepant		
Valproate	Anti-epileptic drugs (decrease neuronal activity by increasing Cl- influx, decreasing Ca++ influx, and other mechanisms)	Inhibit activation of brainstem
Topiramate		
Gabapentin		

Table 1: Common Medications for Anti-migraine Treatment[4]

Acute attack treatment is divided into prodromal phase and headache phase. The prodromal phase is described by aura, neurological symptoms, that are experienced prior to the actual headache[26]. Treatment applied during the prodromal phase is conducted by the Triptan family, which includes drugs such as Zolmitriptan, Naratriptan, Rizatriptan, Eletriptan, Almotriptan, and Sumitriptan, which have been shown to quickly decrease migraine headaches in patients[2]. Sumatriptan is the most widely used antimigraine drug, and is the most effective antimigraine prophylactic drug. This drug brings elevated CGRP back to normal levels and also relieve the headache in studies that have been conducted[43]. Triptan oral administration inhibits gastrointestinal mobility and thus may not completely relieve pain, and as such various other methods of administration such as nasal spray or subcutaneous injection may be preferred[44].

Triptans have been found to be more effective when administered during the headache phase rather than during the aura phase[45]. In fact, the general oral treatment of migraine attacks is recommended by the European Federation of Neurological Sciences to be earlier during the headache phase to prevent incorrect absorption that may take place during the migraine[46]. But if a non-oral administration of triptans is being given, the most effective period to administer the drugs has been found to be later, often prior to the symptoms of the migraine becoming severe[48]. A side effect of triptan drugs is that they are able to constrict coronary arteries, leading to chest tightness and pain, which can be an issue in patients suffering from coronary diseases[49].

Also given during the prodromal phase is the vasoconstrictor Dihydroergotamine, which is a derivative of ergotamine. This drug shouldn't be used while pregnant or by

patients that have coronary artery disease because nausea is a side effect among many others. The triptan drugs have been shown to be more effective in most cases[50].

The headache phase is associated with cerebral vasodilation as well as symptoms that include nausea or vomiting, and analgesics such as non-steroid anti-inflammatory drugs are used for treatment[26]. These aren't specific and act on many different receptors and molecules such as cyclooxygenase and other inflammation related receptors. Naproxen, meclogenamate, and aspirin are common NSAIDs used for antimigraine treatment[50]. If the pain is severe, opioids, mepreidine, or codeine sulphate can be used to decrease pain. Opiates specifically decrease the calcium influx (pre-synaptic) and increase the potassium efflux (post-synaptic), which then decreases the duration of the action potential by decreasing the positive charge inside the postsynaptic terminal. Anti-emetic drugs are used to treat nausea, examples of which are domperidone and metoclopramide[51]. Medication overuse is lower in patients that are using triptan rather than analgesics, and opioids have been shown to have a lower efficiency at treating migraines overall[52].

Epilepsy and migraines have similar clinical features, and as such antiepileptic drugs can also be used for antimigraine treatment due to their prevention of the stimulation of the brainstem[4]. Topiramate, gabapentin, and valproate are involved with gamma-aminobutyric acid by increasing the inhibition of GABA. Gabapentin and valproate alter GABA metabolism, leading to its eventual inhibition[53]. Topiramate also inhibits the action of GABA, but does so by acting on the receptors. In various experiments conducted, the actions of these drugs have been shown to be more

positive than of a placebo[54]. Topiramate has also been shown to prevent the exocytosis of CGRP and thus prevent vasodilation as well by directly acting on trigeminal sensory nerves[55].

For a severe migraine attack, the first drugs of choice are sumatriptan administered subcutaneously and acetylsalicylic acid administered intravenously. Steroids can help treat a status migrainosus. Betablockers, topiramate, valproic acid, and flunarizine are the first choice to treat the prophylaxis of migraines. Second choice drugs are bisoprolol, naproxen, petasites, and amitriptyline[51].

CGRP Related Treatment

Focus has been given to attempt to block CGRP release and/or antagonizing and blocking its receptors[26]. The reason for this recent focus has been to prevent vasodilation and aim to treat migraines effectively. When the superior sagittal sinus is electrically stimulated, Intravenously administered BIBN4096BS (olcegepant) has prevented trigeminocervical complex-evoked activity. Olcegepant, a CGRP receptor antagonist that has affinity for CGRP receptors in humans, and it inhibited CGRP's associated effects[56]. This displays that a CGRP receptor antagonist, in particular this drug, can effectively treat migraine headaches[57]. In trials that were conducted, it didn't constrict coronary arteries, providing an advantage over triptans[54].

BIBN4096BS has also been found to lower the activity of neurons that have meningeal input and block an increase of facial blood flow[58]. Thus, potential candidates for migraine treatment display combined peripheral and central action in the trigeminal

nucleus. This is especially apparent in cardiovascular disease patients, on whom triptan effects are limited due to vasoconstrictive activity on coronary arteries[59].

Telecagepant (MK-0974) is another CGRP receptor antagonist that is effective when orally administrated. If given in the dose of 300 mg, it has been shown to decrease pain and symptoms two hours after ingestion[69]. Both olcegepant and telecagepant have been used in phase II clinical trials, and telecagepant is currently in its phase III clinical trials[65]. Both of these CGRP antagonists have been effective at decreasing the pain associated with migraines, and have done so without vasoconstrictive activity. However, increased levels of these two CGRP receptor antagonists have been associated with liver toxicity, and this has limited their use[60].

Another CGRP receptor antagonist that has been effective so far at decreasing pain associated with migraines is B144370TA. This receptor antagonist is in its phase II clinical trials, and has not been associated with vasoconstrictive activity or even liver toxicity, but more research needs to be conducted[61].

Topiramate, an anti-epileptic drug, is another antimigraine treatment option that blocks the release of CGRP, and thus stopping vasodilation in trigeminal neuron[60]. The method of action of topiramate has been seen to be more presynaptic than postsynaptic since topiramate can stop the release of CGRP from the trigeminal neurons that are responsible for the release[63].

LABORATORY METHODS

There have been many animal models developed to mimic the headache to analyze pathomecahsims of migraine and examine the effects of antimigraine drugs. Antimigraine drugs mainly aim to constrict cranial blood vessels that have been dilated, and also block vasoactive substance, such as neuropeptide, release in order to prevent neurogenic inflammation[26]. In pigs, the carotid arteriovenous anastosmes have been constricted while in rats the superior sagittal sinus or the trigeminal ganglion has been electrically stimulated. In rats, the sensory nerve fibers have been chemically stimulated by capsaicin to mimic a painful condition, which is similar to the experiment that has been conducted by the author. Different vasodilators have been used to bring about a headache that is vascular in nature, examples of which are nitroglycerin, prostaglandin E1, histamine, and also CGRP[38].

Peripheral nerves can be electrically stimulated to bring about pain, and the trigeminal ganglion (TG) can be stimulated to serve as a model the for migraine. Electrical stimulation in rats of the trigeminal nerve leads to extravasation of plasma proteins in the territory of the trigeminal nerve innervation[39]. This extravasation has been proposed because of the release of neuropeptides, possibly CGRP and SP[40].

In experiments where the trigeminal ganglion was stimulated unilaterally in the rat, much greater swelling of the nerve fibers and terminals is observed, around four times greater than the normal size. The electrical stimulation was conducted with the parameters 5 Hz, 5ms, 0.1-1 mA for 5 minutes[41]. A greater level of CGRP immunohistochemical staining was also observed, providing more evidence for CGRP's

effect in migraines[41]. After the trigeminal ganglion was electrically stimulated for thirty minutes, the nerve terminals of the fibers appeared to be disintegrated and collapsed, which can be attributed to the exocytosis of the neuropeptides into blood vessels that were present in the nerve terminals[42].

In line with this, an experiment showed that in the superior sagittal sinus, CGRP levels increased after the trigeminal nerve was electrically stimulated, but this increase wasn't as great when an antimigraine drug such as sumatriptan was used[43]. When sumatriptan is intravenously administered before electrical stimulation of the trigeminal nerve, the collapse of the nerve terminals was prevented and CGRP instead accumulated in the terminals, showing the blockage of its release by anti-migraine drugs[44]. When the trigeminal nerve is stimulated, the neuropeptide content is released centrally and peripherally because CGRP-immunoreactivity is decreased on the backside of the trigeminal nucleus on the stimulated side[45]. Meanwhile, the expression of an activating gene, the c-fos oncoprotein is increased in the caudal trigeminal nucleus, further providing evidence for the neurotransmitter release centrally from the trigeminal terminals[2].

Experiment Conducted for this HIM Project

An ex-vivo experiment was conducted in Dr. Kiminobu Sugaya's lab to examine the dura mater of normal mice when capsaicin, a chili pepper constituent, is added to stimulate sensory nerves. We attempted to visualize CGRP Immunoreactive sensory nerve fibers, similar to the Figure 1 that was obtained from an in-vivo experiment conducted in rats in 1997[63]. This experiment focused on the visualization of the nerve

fibers and vessels since in normal conditions, these nerve fibers and blood vessels are present together. The fibers come mainly from the trigeminal ganglion, and are either perivascular or found as free nerves in the rat dura mater[63]. This picture shows the CGRP expressing fibers in the rat dura mater surrounding a blood vessel.

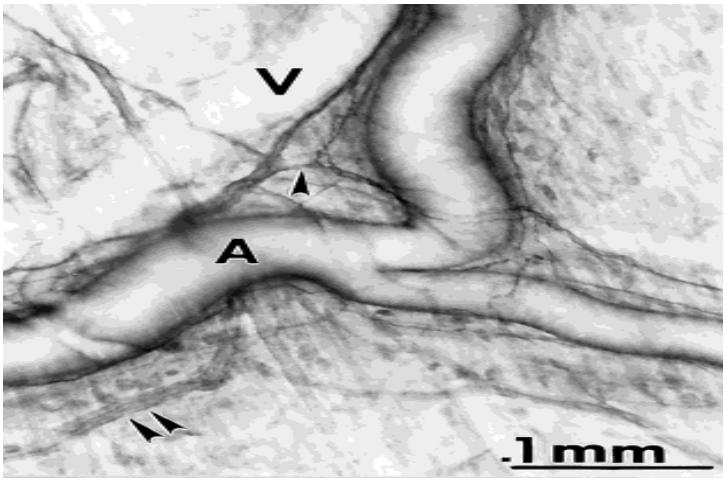

Figure 1: CGRP Immunoreactive Sensory Nerve Fibers[63]

In the experiment conducted, seven mice were euthanized under deep anesthesia, and then were decapitated. Their skull was dissected and the dura mater, which is the outer meninges of the brain, was removed and cut into 2-3 pieces (Figure 2). Five mice were used for the experiment, and two mice were used as practice for extraction of the dura mater.

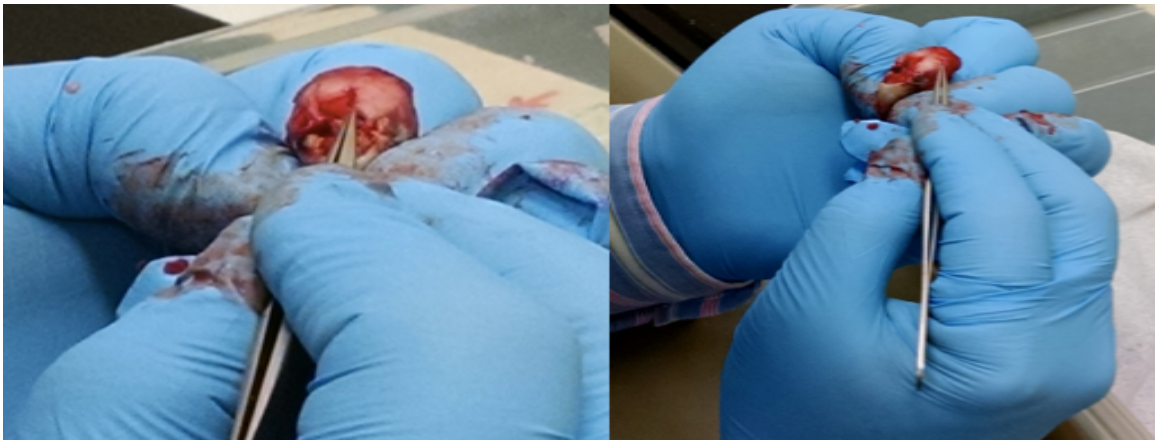

Figure 2: Removal of Mice Dura Mater

The dura mater was placed in cell culture medium in well plates (Figure 3). The well plates were placed in an incubator at 37 degrees Celsius with 5% CO_2 to mimic living tissue conditions and perform an ex-vivo experiment (Figure 4).

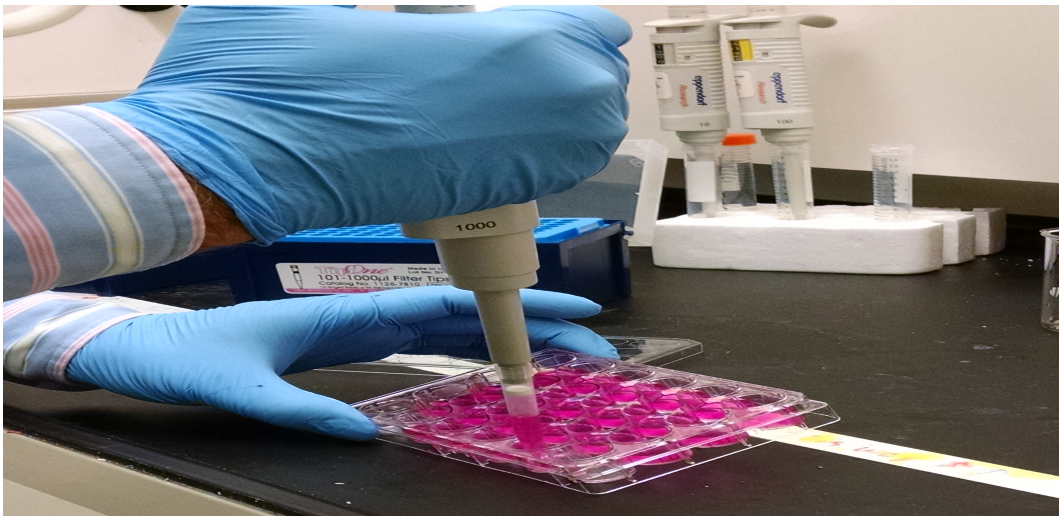

Figure 3: Addition of Dura Mater to Well Plate Containing Cell Culture Medium

Figure 4: Dura Mater in Well Plates Prior to Incubation

The dura mater was incubated with rabbit polyclonal anti-CGRP antibody at 1/100 concentration for 3-4 hours, which is expected to bind to CGRP, both in the nerve fibers and outside the fibers, wherever CGRP is present. This also served as a visualization technique because this primary antibody is already conjugated to FITC, which helps in analyzing the tissue under fluorescent microscopy. Each of the dura mater obtained from the five mice were separated into five different groups, each of which aimed to present a different scenario than the other. Of the total 5 groups, 2 were controls: there was both a negative control and a positive control.

Capsaicin was used to stimulate sensory nerve fibers and mimic a painful condition. This would lead to activation of the nerve fibers and release of neurocontents including CGRP from those nerve fibers. The dura mater was treated with CGRP

antibody to neutralize it and block its function. It also helped in determining where CGRP is located, either inside or outside the nerve fibers. For immunohistochemistry, the unspecific antibodies were not blocked. Using serum against un-specific antibodies may have increased the visualization hence better results, but by blocking the un-specific antibodies, the in-vivo conditions mimicked in ex-vivo conditions. The CGRP receptor antagonist that was added aimed to bind to specific CGRP receptors on the nerve fibers and blood vessels and prevent the release of CGRP, and thus prevent vasodilation from theoretically occurring.

In the experiment, two groups were set as controls, and three groups were experimental groups. A description of the five groups and what was added to each group is given in Table 2.

Group-1	Group-2	Group-3	Group-4	Group-5
1-Only CGRP-Ab For 3-hrs. 2- Antibody was removed and medium was added for total of 4-hrs in the incubator on shaker, at 37^{O}C	1-First Capsaicin for 45 min 2- CGRP-Ab For 3:15-hrs For total of 4-hrs in the incubator on shaker, at 37^{O}C	1- First CGRP-Ab For 3:15-hrs 2- then Capsaicin for 45 min For total of 4-hrs in the incubator on shaker, at 37^{O}C	1- First CGRP-Ab + CGRP antagonist for 3:15-hrs 2- then Capsaicin for 45 min For total of 4-hrs in the incubator on shaker, at 37^{O}C	1- First CGRP antagonist for 1:15 hours 2- then Capsaicin for 45 min 3- CGRP-Ab for 2-hrs For total of 4-hrs in the incubator on shaker, at 37^{O}C

Table 2: The Five Groups of Dura Mater with Respective Additions

The negative control had CGRP antibody added and was then incubated for 2 hours. It was then washed with PBS the next day and was analyzed. Without capsaicin, the nerve fibers weren't stimulated, which would mean that the release of CGRP wasn't

induced. The CGRP antibody was added to detect CGRP expressing neuro-fibers in the dura mater. The dura mater had been placed in the cell culture medium and incubated for 2 hours with CGRP antibody at 37 degrees Celsius, and then transferred into 4 degrees Celsius overnight and washed with PBS then next day and transferred onto glass slides for fluorescent microscopy, and after everything was stained to see the fibers. As such the final results of the microscopy for the negative control (Figure 5) just faintly display the nerve fibers as a result of the staining.

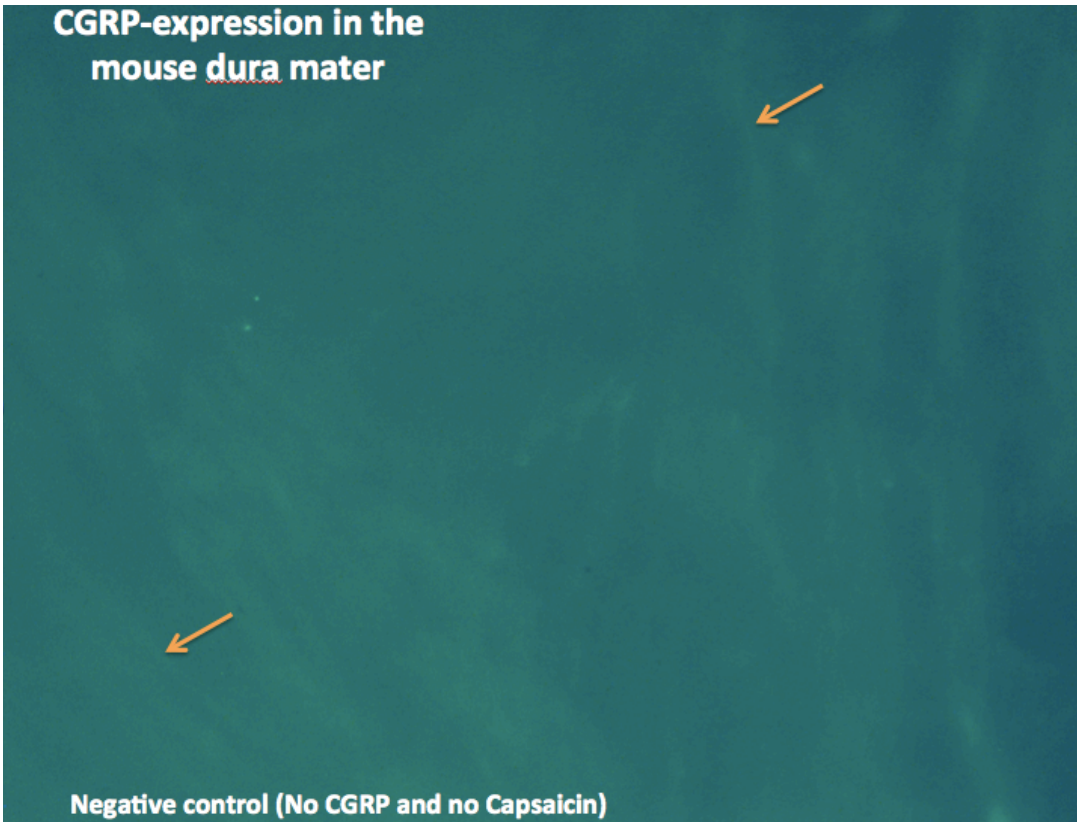

Figure 5: Group 1: Negative Control

The positive control (Figure 6) had capsaicin added first to stimulate the nerve fibers for 45 minutes and then the CGRP antibody was added for 3 hours and 15 minutes in 37 degrees Celsius in the incubator. Similar results were obtained compared

to the negative control in which visualization wasn't completely ideal, but nerve fibers were faintly visible.

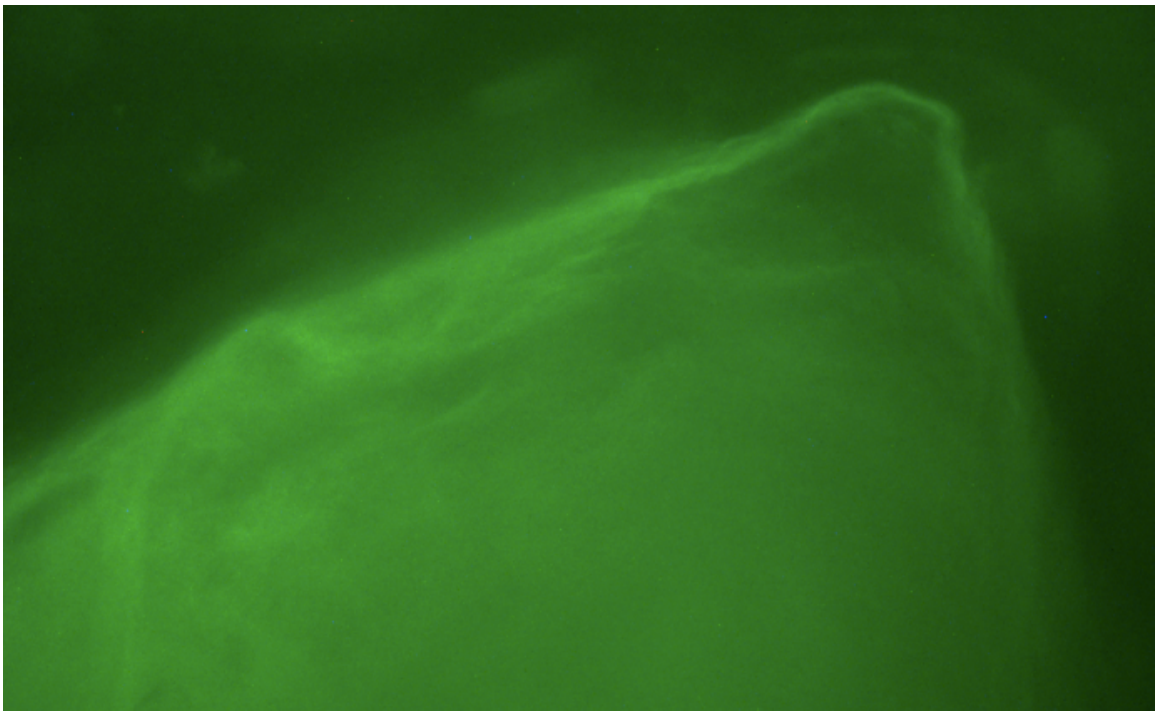

Figure 6: Group 2: Positive Control (Capsaicin and CGRP Antibody)

In the first experimental group, the CGRP antibody was added to the dura mater and incubated for three hours so that it would bind to the CGRP and help in visualization of wherever CGRP is. CGRP-antibody was added for 3 hours and 15 minutes in 37 degrees Celsius on the shaker, and after incubation capsaicin was added fro 45 minutes. This is, in fact, is displayed in the results (Figure 7), where CGRP has been released and the fibers aren't visualized as well because there's minimal amount of CGRP left in the fibers. If there had been CGRP present in the fibers, then that would've meant that the fibers would be much brighter. This means that capsaicin had caused the excitation of the nerve fibers, and the release of the content (CGRP).

Figure 7: Group 3: Nerve Fibers after CGRP Antibody and Capsaicin were added

In the second experimental group, capsaicin, the CGRP receptor antagonist, and the CGRP antibody were all added to the dura mater. Capsaicin stimulates the nerve fibers, which leads to the release of vasoactive substances of CGRP, which can be visualized by the CGRP antibody that is conjugated to FITC. However, since the CGRP receptor antagonist was also added, this would block the receptors on the nerve fibers and prevent the release of CGRP. Since the nerve fibers are stimulated, CGRP is expected to be present, but CGRP would be expected to be in the nerve fibers rather than released because the receptor antagonists are blocking the exocytosis of CGRP. Thus, the greatest visualization of the nerve fibers is expected in this experimental group since CGRP is present in the fibers. In this case, the CGRP antibody was placed together with the CGRP receptor antagonist on the dura mater for 3 hours and 15 minutes, and then the nerve fibers were challenged with capsaicin for 45 minutes. The fibers are much clearer (Figure 8), which means that the antibody was effective and the

receptors were blocked (which are present everywhere on the vessels and on the fibers), meaning CGRP wasn't released.

Figure 8: Group 4: Nerve Fibers after Capsaicin, the Antibody, and the Antagonist were added

The last experimental group had the CGRP receptor antagonist added with the capsaicin onto the dura mater (Figure 9). Capsaicin is expected to stimulate the nerve fibers and lead to the release of CGRP, but since the antagonist is also present, this would lead to the blocking of the receptors involved in the release of CGRP. Thus, we would expect the same results as the second experimental group. The CGRP antagonist was added for around an hour and 15 minutes. Then Capsaicin was added fro 45 minutes, followed by the CGRP antibody for 2 hours.

Visualization for all of the results had been achieved by transferring the pieces of dura mater onto a glass slide, and then adding glycerol/PBS medium. The slides were then covered and analyzed using a fluorescent microscope.

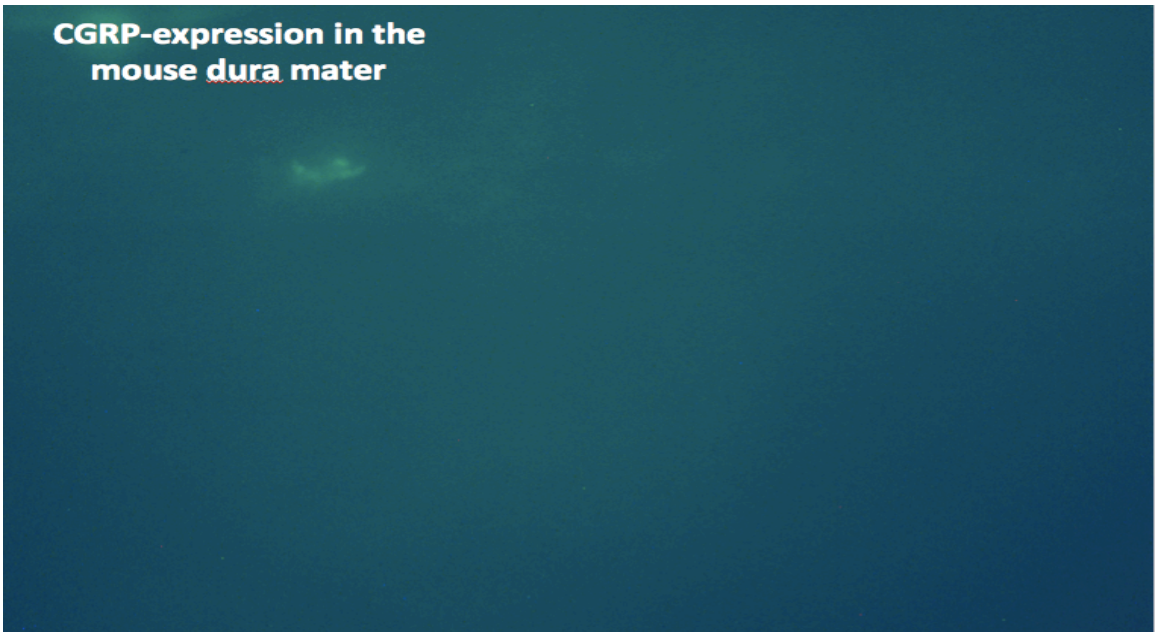

Figure 9: Group 5: Nerve Fibers after CGRP Antagonist, Capsaicin, and then CGRP Antibody were added

Issues that were experienced in the experiment were the difficult of working with the dura mater of mice. It's difficult to correctly remove the dura mater, and is quite time consuming. In addition, mouse tissue is thin and narrow, and it may be difficult to find thick fibers that are desired for ideal results.

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